Differential activity of a variant form of the human Id-1 protein generated by alternative splicing

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Abstract Members of the Id family of helix-loop-helix proteins are ubiquitously expressed and dimerize with members of the class A and class B basic helix-loop-helix proteins. Due to the absence of a basic region, Id proteins act as dominant-negative antagonists of basic helix-loop-helix transcription factors, which regulate cell growth and differentiation in diverse cell types. Recent findings suggest that the functions of Id proteins are well regulated at both the transcriptional level and the post-transcriptional level. We show here that the alternative splicing variant of human Id-1 protein possesses a different binding specificity for basic helix-loop-helix transcription factors and is expressed in a cell cycle-dependent manner. Therefore, alternative splicing of Id-1 could provide a post-transcriptional mechanism to regulate Id-1 function.

Key words: Transcription factor; Helix-loop-helix; Id; RNA splicing; Cell cycle

1. Introduction

The helix-loop-helix (HLH) transcription factor Id plays important roles not only in suppressing cellular differentiation but also in promoting cell growth and apoptosis [1]. Members of the Id family proteins, Id1–Id4 in mammalian cells, have highly homologous HLH domains but lack the basic DNA binding region [2–10]. Thus, Id proteins bind to basic-helix-loop-helix (bHLH) transcription factor though the HLH domain and heterodimerization with bHLH proteins renders the bHLH protein incapable of binding to the E-box sequence (CANNTG) of DNA. Therefore, Id proteins are considered to be a dominant negative HLH transcription factor. In general, Id proteins are highly expressed in growing cells and their expression is down-regulated upon differentiation in many cell types. Several lines of evidence also suggest that Id proteins play a role in the G0 to S phase transition of the cell cycle. Stimulation of quiescent fibroblasts with growth factors induces the transcription of Id1, Id2 and Id3 [4,6,7]; inhibiting Id protein synthesis by antisense oligonucleotides prevents the re-entry of arrested cells into the cell cycle [7,11]; and Id expression declines as senescent fibroblasts lose their proliferative activity [7].

Although Id proteins are transcriptionally well regulated during the progression of the cell cycle, recent findings demonstrated that Id proteins are also regulated by post-transcriptional mechanisms. Id-2 and Id-3 proteins share a conserved, functional phosphorylation site for cyclin E and cyclin A-CDK2 at Ser^5 [12,13]. Phosphorylation of Id-2 and Id-3 proteins by CDK2 alters their binding specificity in the late G1 to S phase of the cell cycle [1]. CDK2 is believed to be an important kinase in the regulation of the G1 to S phase transition in the mammalian cell cycle [14,15]. Therefore, these lines of evidence provide a link between CDKs and bHLH transcription factors that may be critical for the regulation of cell proliferation and differentiation. A potential CDK2 phosphorylation site is present at the same position in Id-4, intriguingly, not in Id-1 (for a review, see [1]). Id-1 is known to have an alternative splicing variant in human, Id-1′ [7], and in mouse, Id-1.25 [16,17].

In this report, we describe the expression pattern and functional activity of both human Id-1 and its alternative splicing product, Id-1′. We show here that the expression patterns of both Id-1 mRNAs are very similar, although the level of Id-1′ mRNA is 5 times lower than that of Id-1 throughout the cell cycle in human primary fibroblasts. Moreover, the binding specificity of Id-1 and Id-1′ to bHLH proteins is significantly different. These results suggest that, although Id-1 is not regulated by CDK2, the activity of Id-1 is post-transcriptionally regulated by alternative splicing.

2. Materials and methods

2.1. Analysis of Id-1 mRNA

Early passage human diploid fibroblasts (HDF), TIG-3 cells, were cultured in low serum (0.2%) medium for 4 days, and then cells were treated with high-serum medium (20%). Total RNA was prepared by extraction in guanidium isothiocyanate, phenol, and chloroform [18] with TRISOL (Gibco-BRL). RT-PCR was performed using a Super-Script preamplification system (Gibco-BRL). The forward (F) and reverse (R) primers for Id-1/Id-1′ were ACCCTGCCCCAGAACCG-CAAG (Primers F), TTTCCAGGCTCCTTAGGCAC (Primer R1) and TTGTTCTCCCTCAGATCCG (Primer R2). The 5′ and 3′ primers for G3PDH were ACCACAGTCCATGCCATCA (Primer G3PDH 5′) and TCCACCACCCTGTTGCTGTA (Primer G3PDH 3′).

2.2. Electrophoretic mobility shift assays

Unlabeled proteins were synthesized by couples transcription and translation of plasmid DNA using the TNT expression system (Promega). Samples (5 μl) of the different translation reactions were mixed and used for DNA-binding assays. An E-box consensus sequence (CANNTG) from the muscle creatine kinase gene enhancer was used in all DNA-binding reactions. Two complementary oligonucleotides, 5′-GGATCCCCCCAACACCTGCTGCCTGA-3′ and 5′-TC-AGGCAGCAGGTGTTGGGGGGAT-3′, were annealed and labeled with [α-^{32}P]dCTP using the Klenow fragment of *E. coli* DNA polymerase. Two equivalent oligonucleotides containing a mutated E-box sequence, i.e. 5′-GGATCCCCCCAAACTGGTCTGCCTGA-3′, were used as competitors in some experiments. DNA binding reactions were done in a total volume of 20 μl containing 20 mM HEPES,

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Abbreviations: HLH, helix-loop-helix transcription factor; bHLH, basic-helix-loop-helix transcription factor; HDF, human diploid fibroblasts; EMSA, electrophoretic mobility shift assay; CDK, cyclin-dependent kinase; FACS, fluorescence activated cell sorter

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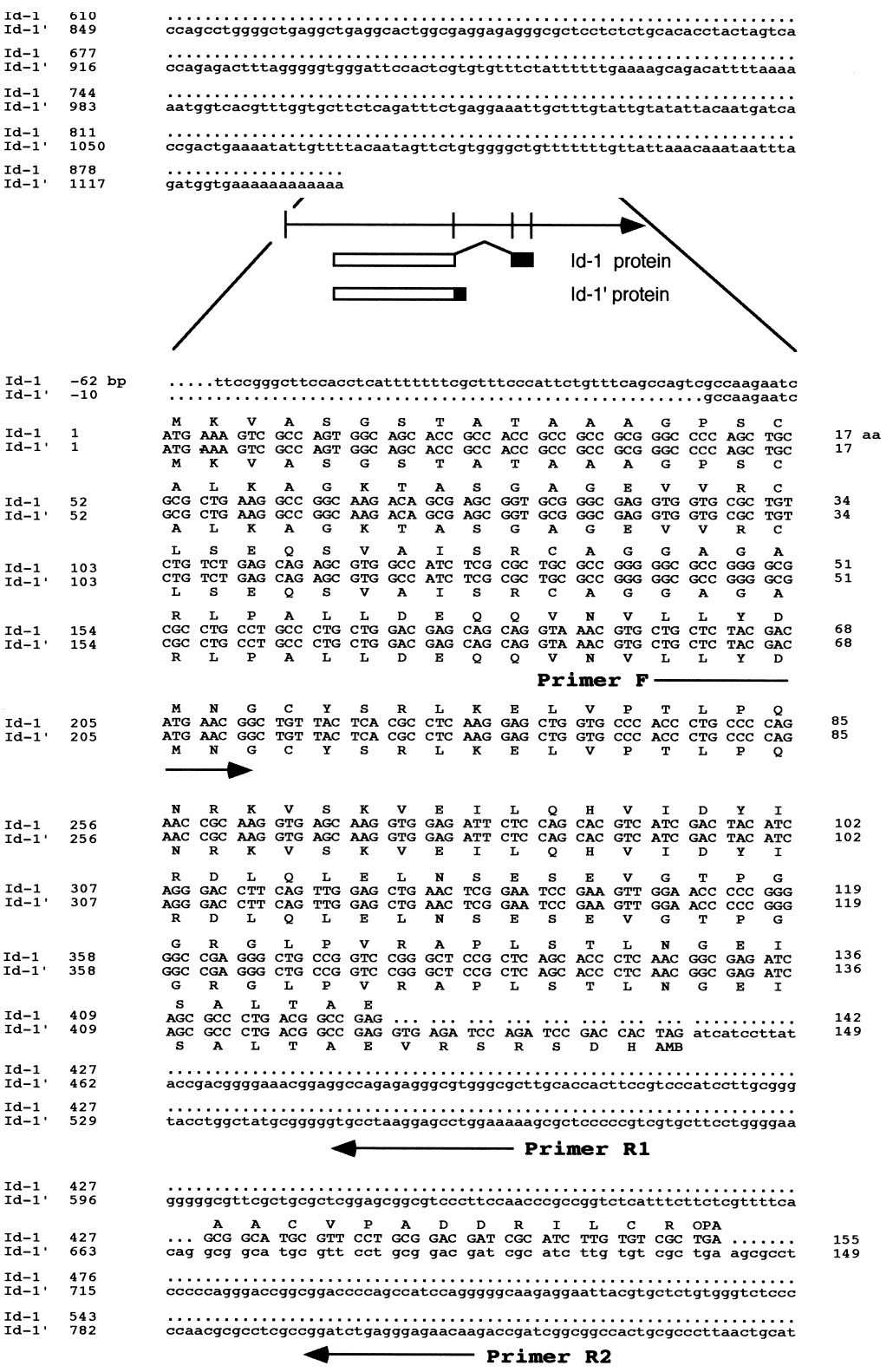
Id-1 protein
Id-1' protein
Primer F
Primer R1
Primer R2

pH 7.6, 50 mM KCl, 1 mM dithiothreitol, 1 mM EDTA, 5% glycerol, 1 μg of soluble-stranded poly(dI:dC) and 0.2 ng of labeled double-stranded probe, at room temperature for 10 min, and for a further 15 min at room temperature after addition of the labeled probe. The binding reactions were then subjected to electrophoresis in a 6% polyacrylamide gel in 25 mM Tris base, 25 mM boric acid and 0.5 mM EDTA (0.5× TBE) at 150 V for 2 h at room temperature. The gels were dried and the labeled complexes were detected by autoradiography.

2.3. Purification of bacterially expressed Id proteins

The human Id-1 and Id-1′ coding sequences were cloned into the pRSET-B vector (Invitrogen) for expression in bacteria. The recombinant proteins were expressed in *Escherichia coli* BL21(DE3)pLysS. Histidine-tagged Id proteins produced in the pRSET-B vector were purified on nickel-charged chelating agarose as recommended by the supplier (Invitrogen).

3. Results and discussion

We previously identified an alternative splicing variant of the human Id-1 gene, Id-1′ [7]. Our previous work suggested that Id-1 mRNA is barely detectable in quiescent early passage HDF; serum coordinately induced mRNA, with two peaks of expression in early and late G1 [7]. However, due to its genomic organization, Id-1′ mRNA is difficult to distinguish from Id-1 mRNA by Northern blot analysis (Fig. 1) [19,20]. Therefore, we performed an RT-PCR analysis using specific primers. Fluorescence activated cell sorter (FACS) analysis suggests that the peak of S-phase is around 24 h after serum stimulation. As shown in Fig. 2, lane 1, both cDNAs corresponding to the Id-1 and Id-1′ are undetectable in quiescent HDF, TIG-3 cells, but are induced about 20-fold within 2 h of serum stimulation. They then decline as the cells progress through G1, and rise again around the S phase. Although the level of cDNA corresponding to Id-1′ was 5 times lower than that of Id-1, Id-1′ showed a similar expression pattern during the progression of the cell cycle. This is consistent with the numbers that were obtained when we cloned Id-1 and Id-1′ cDNA clones from a TIG-3 cell cDNA library [7]. PCR products were confirmed by Southern blot analysis using a ^{32}P-labeled Id-1 cDNA probe (data not shown). Moreover, synthesized cDNAs were also subjected to PCR using G3PDH primers as a control (Fig. 3, bottom panel). The same results were obtained with two different combinations of PCR primers (F/R1 and F/R2), suggesting that our RT-PCR assay was reliable (Fig. 2, top panel and middle panel).

Although the amino acid sequence of human Id-1 is very similar to that of mouse Id-1, there is only a limited homology between the C-terminal region of human Id-1′ and that of mouse Id-1.25 (Fig. 3). Therefore, we examined the functional activity of human Id-1 and Id-1′ by the electrophoretic mobility shift assay (EMSA) as previously reported [12]. A ^{32}P-labeled MCK-E-box DNA probe was incubated with a 1:1 mixture of ΔE12 (a partial fragment of the E12 protein that includes the bHLH region (residues 508–654)) and MyoD protein which were translated in vitro. As we have previously

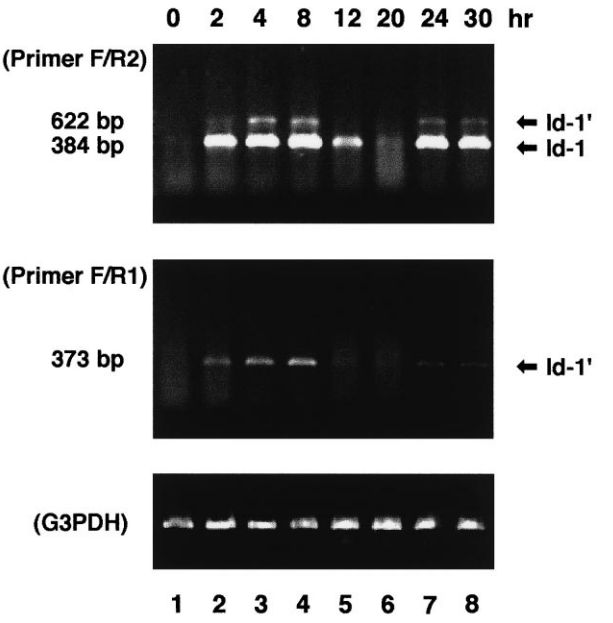

Fig. 2. Comparison of mRNA expression between Id-1 and Id-1′. Quiescent TIG-3 cells were stimulated by the addition of serum and total RNAs were prepared at the indicated times after serum addition. One microgram of total RNA was used for RT-PCR using different combinations of PCR primers. Primers F and R2 (top panel); Primers F and R1 (middle panel); Primers for G3PDH (bottom panel).

reported, two DNA binding complexes were observed: one consists of a ΔE12 homodimer (lower band) and the other consists of a heterodimer of ΔE12 and MyoD [12]. Addition of an excess amount of unlabeled MCK-E-box oligonucleotide completely abolished the formation of complexes on the labeled probe, whereas an oligonucleotide containing a mutated E-box sequence did not (see previous report [12]). To examine the activities of the Id-1 and Id-1′ proteins, we added different amounts of recombinant Id proteins to the mixture of the ΔE12 and MyoD proteins. Approximately 0.5–1 ng of recombinant Id-1 protein was enough to inhibit the formation of a ΔE12-MyoD-DNA complex (Fig. 4, lanes 3 and 4) whereas 5- to 10-fold more Id-1′ protein (∼5 ng) was required to obtain the same effect on the heterodimer (Fig. 4, lane 11). In contrast, the addition of 20 ng of Id-1′ protein was able to inhibit the formation of the homodimer ΔE12-ΔE12-DNA complex, whereas disruption of the homodimer complex was not observed with 20 ng of Id-1 protein (Fig. 4, lanes 7 and 13). Similar EMSA assays were then performed using ΔE12 alone. Recombinant Id-1 and Id-1′ proteins were added to the ΔE12 protein. As shown in Fig. 5, the addition of 5 ng of Id-1′ protein inhibited the formation of the homodimer ΔE12-ΔE12-DNA complex and 25 ng of Id-1′ protein completely abolished the complex formation (Fig. 5, lane 7). However, 5-fold more Id-1 protein was required to obtain the same effect on the homodimer (Fig. 5, lanes 4 and 6). A similar difference

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Fig. 1. Organization of the human Id-1/Id-1′ gene. The map shows the 7.5-kb fragment containing the human Id-1/Id-1′ gene, indicating the positions of the TATA box and polyadenylation sites. Shown below the map are the exons (small solid bars) and an intron, the Id-1/Id-1′ pre-mRNA, and the Id-1 and Id-1′ proteins (large open square bars) which differ at the C-terminus due to differential splicing of the intron. The cDNA sequence and predicted amino acid sequence of the two isoforms of human Id-1 and Id-1′ are shown below the map. The DNA sequences that were used for the RT-PCR analysis are shown as Primers F, R1 and R2.

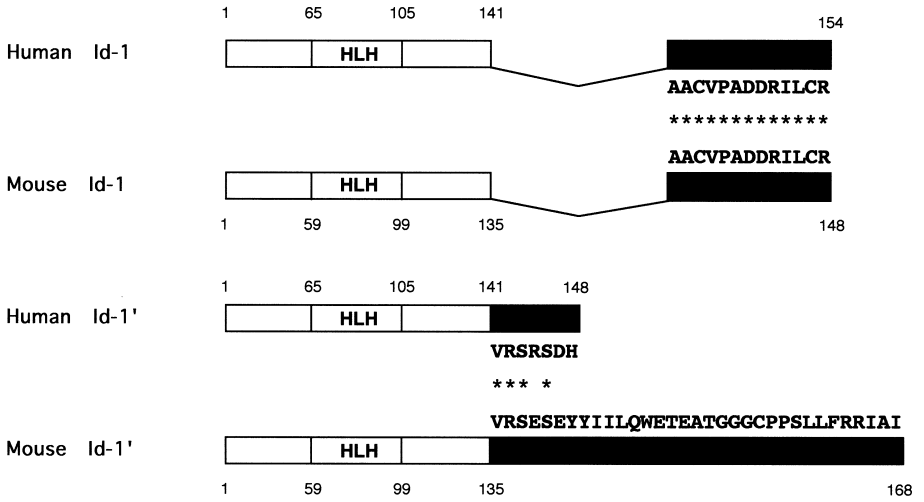

Fig. 3. Schematic representation of the structure and predicted amino acid sequences of Id-1 and the spliced variant Id-1′. The relative positions of the regions common to Id-1 and Id-1′, encompassing the HLH domain, are depicted by open boxes, and the amino acid sequences of the carboxyl termini unique to each protein are shown.

was observed between Id-1 and Id-2 proteins in our previous report [12]. Although Id-1′ protein is identical to Id-1 protein except for the C-terminal 13 amino acids, the activity of Id-1′ protein is very similar to that of Id-2 protein [12]. These results indicate that the specificity of Id-1 for binding to bHLH protein could also be regulated by the C-terminal region of the Id proteins. Therefore, alternative splicing might be an important mechanism for regulating the binding specificity of Id-1 protein. It has been suggested that Id-1 protein also binds to a novel protein, MIDA1, which does not have the bHLH motif [21]. Thus, it would be interesting to know how Id-1′ binds to MIDA1.

The data presented here suggests that although Id-1 is not regulated by CDK2, the activity of Id-1 is regulated by alternative splicing. Deed et al. [22] reported similar results for Id-3 and its alternative splicing variant, Id-3L. Therefore, alternative splicing could be an important mechanism in the regulation of the functions of Id proteins.

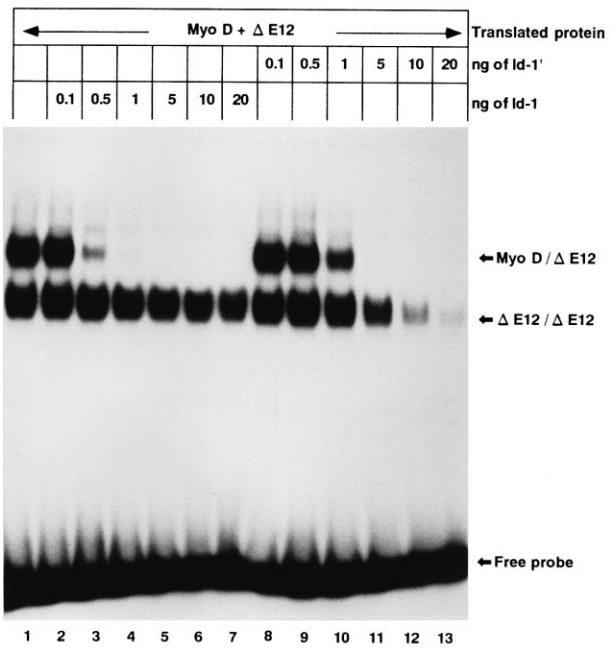

Fig. 4. Differential activity of Id-1 and Id-1′ proteins. The ΔE12 form of E12 and MyoD were synthesized by in vitro translation and tested for the ability to bind to the MCK promoter E-box oligonucleotide in an electrophoretic mobility shift assay. The positions of the free ^{32}P-labeled probe and homo- and heterodimeric complexes are indicated on the right (lane 1). A similar assay was performed using ΔE12 and MyoD together with 0.1, 0.5, 1.0, 5, 10 or 20 ng of bacterially synthesized, histidine-tagged Id-1 or Id-1′ protein (lanes 2–13).

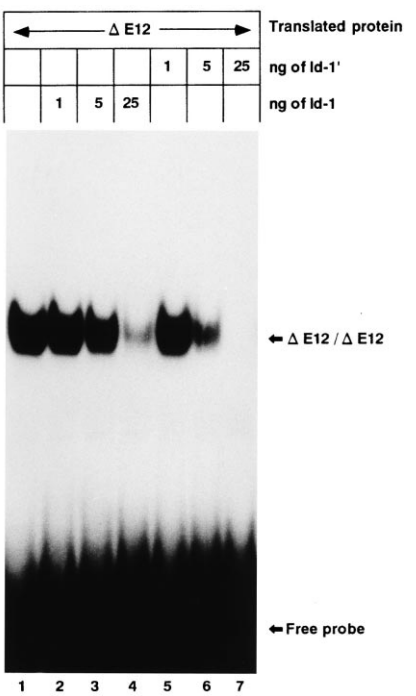

Fig. 5. Effects of Id-1 and Id-1′ proteins on ΔE12 homodimeric bHLH-E box binding. EMSA was performed using in vitro translated ΔE12 protein, and complexes were challenged with increasing inputs of bacterially synthesized, histidine-tagged Id-1 or Id-1′ proteins. The positions of the free ^{32}P-labeled probe and homodimeric complexes are indicated on the right.

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